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(54) PROCESS FOR PREPARING INJECTABLE FLUOROCARBON EMULSION CAPABLE OF CARRYING OXYGEN

ERRATUM

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patients suffering from severe bleeding.

Heretofore, it has been the ordinary expedient for life saving in the case of human bleeding where the loss of blood is not more than 1500 ml, to supply a transfusion containing a substance having a colloidal osmotic pressure, for example, dextran, or an electrolyte solution such as Lactate Ringer's solution, in order to prevent bleeding shock. However, where the loss of blood exceeds 1500 ml, the amount of oxygen carried by red blood-cells in blood becomes short, and tissue respiration at the peripheral tissues becomes insufficient. Therefore, life saving has been impossible in these cases unless blood transfusion is conducted.

Preparations having an ability to carry oxygen in animal bodies have been studied by a number of people, but it is only in recent years that the preparations have been shown to have a life saving effect when injected into animals. In 1966, L. C. Clark Jr. succeeed in keeping mice living for a long time by immersing the mice in some kind of fluorocarbon solutions (see Science, *152*, 1755 (1966)), and studies on utilization of fluorocarbons as an oxygen carrier in living bodies were started at that time. In 1968, R. P. oxygen, and have developed a novel process for preparing, on a mass-production scale, an injectable emulsion capable of keeping dogs and monkeys living for a long time by blood exchange transfusion.

According to the present invention, there is provided a process for preparing an injectable fluorocarbon emulsion capable of carrying oxygen, which comprises emulsifying in an aqueous salt solution with surfactant a fluorocarbon selected from perfluorobutyletetrahydrofuran, perfluorotributylamine, perfluorooctane, perfluorodecalin, perfluoromethyldecalin and 2-monohydroxy-nonacosafluoro-3,6,9,12 - tetraoxa - 5,8,11 - trimethylpentadecane, the fluorocarbon having an ability to dissolve at least 30 V/V % oxygen under a 100% oxygen atmosphere at an atmospheric pressure, centrifuging the resulting aqueous emulsion to adjust the particle size of fluorocarbon particles in the emulsion to within the range of 0.05μ to 0.25μ, and sterilising the resulting emulsion under rotation.

For example, when 20 W/V % of perfluorotributylamine is made to be contained as the fluorocarbon, the emulsion obtained according to the present invention can contain 12.5 ml of oxygen per liter, but as a result

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(72) Inventors RYOZO WATANABE, TADAKAZU SUYAMA, KUZUMASA YOKOYAMA and YOHEI ODAK

(54) PROCESS FOR PREPARING INJECTABLE FLUOROCARBON EMULSION CAPABLE OF CARRYING OXYGEN

(71) We, GREEN CROSS CORPORATION, a corporation organized under the laws of Japan, of 1,3-chome, Gamaucho, Joto-ku, Osaka, Japan, and TANABE SEIYAKU CO. LTD., a corporation organized under the laws of Japan, of 21 Dosho-machi-3-chome, Higashi-ku, Osaka, Japan, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement: —

This invention relates to a process for preparing an injectable emulsion capable of carrying oxygen, which is used in life saving of patients suffering from severe bleeding.

Heretofore, it has been the ordinary expedient for life saving in the case of human bleeding where the loss of blood is not more than 1500 ml, to supply a transfusion containing a substance having a colloidal osmotic pressure, for example, dextran, or an electrolyte solution such as Lactate Ringer's solution, in order to prevent bleeding shock. However, where the loss of blood exceeds 1500 ml, the amount of oxygen carried by red blood-cells in blood becomes short, and tissue respiration at the peripheral tissues becomes insufficient. Therefore, life saving has been impossible in these cases unless blood transfusion is conducted.

Preparations having an ability to carry oxygen in animal bodies have been studied by a number of people, but it is only in recent years that the preparations have been shown to have a life saving effect when injected into animals. In 1966, L. C. Clark Jr. succeeed in keeping mice living for a long time by immersing the mice in some kind of fluorocarbon solutions (see Science, *152*, 1755 (1966)), and studies on utilization of fluorocarbons as an oxygen carrier in living bodies were started at that time. In 1968, R. P. Geyer reported that total blood of a mouse was exchanged with a fluorocarbon emulsion by blood transfusion and the mouse could be kept living for a few hours ("Organ Perfusion and Preservation" Appleton-Centry Crafts, page 85 (1968)). Then, Clark *et al* reported that total blood of a dog was exchanged with a fluorocarbon emulsion by blood exchange transfusion, and the dog could be successfully kept living for a long time (Chemical and Engineering News, Dec. 15 (1969), page 51).

The present inventors have made studies on a preparation having an ability to carry oxygen, and have developed a novel process for preparing, on a mass-production scale, an injectable emulsion capable of keeping dogs and monkeys living for a long time by blood exchange transfusion.

According to the present invention, there is provided a process for preparing an injectable fluorocarbon emulsion capable of carrying oxygen, which comprises emulsifying in an aqueous salt solution with surfactant a fluorocarbon selected from perfluorobutyletetrahydrofuran, perfluorotributylamine, perfluorooctane, perfluorodecalin, perfluoromethyldecalin and 2-monohydroxy-nonacosafluoro-3,6,9,12 - tetraoxa - 5,8,11 - trimethylpentadecane, the fluorocarbon having an ability to dissolve at least 30 V/V % oxygen under a 100% oxygen atmosphere at an atmospheric pressure, centrifuging the resulting aqueous emulsion to adjust the particle size of fluorocarbon particles in the emulsion to within the range of 0.05μ to 0.25μ, and sterilising the resulting emulsion under rotation.

For example, when 20 W/V % of perfluorotributylamine is made to be contained as the fluorocarbon, the emulsion obtained according to the present invention can contain 12.5 ml of oxygen per liter, but as a result

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of further studies to obtain an emulsion having a higher oxygen content, the present inventors have found that a fluoro-carbon emulsion containing, for example, 69.0 ml of oxygen per liter can be obtained by deaerating said fluorocarbon and the aqueous salt solution under a reduced pressure before the emulsification, blowing oxygen into the deaerated fluorocarbon and aqueous salt solution thereby to dissolve the oxygen therein, then carrying out said emulsification and centrifugal separation and sterilizing the emulsion under the oxygen atmosphere under rotation. It has been found that the fluorescent emulsion having such a high oxygen content is obviously effective in improving the living body just after the loss of blood.

As the non-ionic surfactant of polyoxyethylene-polyoxypropylene system capable of emulsifying and stabilizing said fluorocarbons as an aqueous emulsion, the heretofore well-known surfactants can be used, but in view of the toxicity, molecular weight and emulsion stability, polyoxyethylene-polyoxypropylene copolymers having molecular weight of 5,000 to 15,000 particularly 8,200 to 10,500, are most suitable. Any yolk lecithin or soybean lecithin can be used as surfactants, which contain polyalchol such as glycerol or sorbitol as emulsion stabilizer.

Emulsification is carried out in the following manner. At first, a predetermined amount of the surfactant is dissolved in a suitable aqueous electrolyte solution and an oxygen-carrying material is added thereto. The resulting mixture is stirred in a homoblender or by a propeller stirrer thereby to prepare a crude emulsion. When a small amount of the emulsion is to be prepared, the crude emulsion is further emulsified in a magneto-strictive ultrasonic wave generator, and when a large amount of the emulsion is to be prepared, it is further emulsified in a Manton-Gaulin type, injection emulsifier. The emulsifying conditions for the former case are that, while the crude emulsion is kept at 40°C or less, the ultrasonic wave of 19 KC is given to the crude emulsion for 15 minutes, and those for the latter case are that, while the crude emulsion is kept at 50°C or less, the crude emulsion is injected under a pressure of 140 kg/cm² at the first stage, under a pressure of 500 kg/cm² at the second and third stages, under a pressure of 560 kg/cm² at the fourth stage and under a pressure of 140 kg/cm² at the fifth stage. Fuorocarbon particles of the thus obtained emulsion are distributed in a particle size range of 0.05μ to 1.0μ, when observed by an electron microscope. When the thus obtained emulsion is injected in an animal directly as such, a good result cannot be obtained as shown in Table 3, and it is necessary to restrict the particle size distribution to a narrower range.

To obtain an emulsion of a higher oxygen content, (1) the thus prepared emulsion is deaerated under a reduced pressure, and pure oxygen is blown into the deaerated emulsion, or (2) the fluorocarbon raw material and the aqueous salt solution are deaerated under a reduced pressure before the emulsification, pure oxygen is blown into the deaerated fluorocarbon raw material and aqueous salt solution thereby to dissolve only oxygen therein, and the thus obtained fluorocarbon raw material and aqueous salt solution are emulsified under the oxygen atmosphere according to said emulsifying procedure. Said procedure (1) is applicable to the preparation of a small amount of the emulsion, and oxygen can be contained in the emulsion theoretically at a higher concentration. However, when 100 l or more of the emulsion is to be prepared, said procedure (2) is more efficient. The differences in the oxygen content due to the difference in the procedure are given in Table 1:

TABLE 1

Oxygen content of fluorocarbon emulsion

Sample	Oxygen-replacing procedure	Oxygen content (ml) per l of emulsion
20 W/V % perfluorotributylamine emulsion A	No oxygen replacement or oxygen atmosphere is used	12.5
20 W/V % perfluorobutyl tetrahydrofuran emulsion B	Same as above	17.4
A	Procedure (1). 100 ml of oxygen gas is aerated per liter of the emulsion for one minute	49.6
B	Same as above	68.1
A	Procedure (2)	69.0
B	Same as above	93.5

The present inventors have found that a centrifugal separation is useful for finely dividing the fluorocarbon particles, and it is the first requirement for the present invention to adjust the particle size distribution to a narrower range by the centrifugal operation. A De Laval or Saval type centrifuge is suitable for the centrifugal operation, and it is advantageous in a mass production scale to continuously carry out the centrifugal separation by said centrifuge. In the case of the former De Laval type centrifuge, a type BP15 K is used and the emulsion is passed through the centrifuge at a flow rate of 30 l/hr and a supernatant liquid is collected, while setting the motor and the rotor at 1500 rpm and 900 rpm, respectively. In the case of the latter Saval type centrifuge, the emulsion is passed therethrough at flow rate of 6 l/hr, while setting the centrifuge at 1000 x g. The particle sizes of the fluorocarbon after the centrifugal separation are in a range from 0.05μ to 0.25μ, and an animal test result reveals that the thus prepared emulsion can be satisfactorily used, as shown in Table 2. To obtain an emulsion having a higher oxygen content, said centrifugal operation is carried out in the oxygen atmosphere.

To use the thus prepared emulsion as an injection material safely, it is necessary to sterilize the emulsion. When the emulsion is heated, particles of the emulsion usually start to join together and are aggregated, and consequently the emulsion undergoes phase separation. The present inventors have found that, to sterilize the emulsion without joining together and aggregating the particles, it is useful to slowly rotate the emulsion in a sterilizer. It is the second requirement for the present invention to sterilize the emulsion under rotation. For example, when the emulsion is sterilized at 115°C for 15 minutes while keeping a container for the emulsion at standstill, the emulsion undergoes complete phase separation, whereas when the emulsion undergoes rotation at 12 rpm under the same conditions as above, only slight growth of the particle sizes is observed. For example, the particle sizes of 0.05μ to 0.25μ are increased only to a range from 0.05μ to 0.375μ. When the emulsion is injected in an animal after said rotary sterilization has been effected, the best results can be obtained, as shown in Table 2. To obtain an emulsion having a higher oxygen content said rotary sterilization is carried out under the oxygen atmosphere.

The particle sizes of the fluorocarbon have been determined all from the electron microscope images, and one example of the particle size distribution is shown in Table 2.

TABLE 2

Particle sizes (μ) / Sample	0.05 — 0.25	0.25 — 0.375	0.375 — 0.5	0.5 — 0.75	0.75 — 1.0
Emulsion obtained by ultrasonic treatment	73%	18%	6%	2%	1%
Emulsion obtained by injection emulsification	72%	21%	6%	1%	0%
Supernatant emulsion obtained by centrifuge	100%	0%	0%	0%	0%
Emulsion obtained by rotary sterilization	87%	13%	0%	0 %	0%

The same particle size distribution can be observed in the case of an emulsion having a higher oxygen content.

Test result on a relation between the particle size distribution of the fluorocarbon and ratio of the dead mice to the tested mice is shown in Table 3. In this test, each sample was prepared by mixing 20 W/V % of perfluorotributylamine and 4 W/V % of polyoxyethylene-polyoxypropylene copolymer having a molecular weight of 8,200 with a lactate Ringer's solution. The circulation blood of the mice was exchanged with the sample until the hematocrit value reached 3% or less, and the living state of the mice was observed after 72 hours.

Table 3.

Particle size distribution and ratio of the dead mice

Sample (particle size distribution)	Number of dead mice per number of tested mice
Emulsion obtained by ultrasonic treatment (0.05—1.0μ)	10/10
Emulsion obtained by injection emulsification (0.05—0.75μ)	8/10
Supernatant emulsion obtained by centrifuge (0.05—0.25μ)	0/10
Emulsion obtained by rotary sterilization (0.05—0.375μ)	0/10

To ascertain the effect due to the difference in the oxygen content, the same test as in Example 3 was carried out with the emulsion [A] of Table 1. The result is shown in Table 4, where 20 Wister strain rats (weight: 120—150 g) were employed in place of the mice.

TABLE 4

Oxygen content and ratio of the dead rats

Sample	Oxygen-replacing procedure	Oxygen content (ml/l of the emulsion)	Ratio of dead rat (number) to tested rat (number)
[A]	No oxygen replacement	12.5	6/20
[A]	Procedure (1)	49.6	2/20
[A]	Procedure (2)	69.0	0/20

Now, the present invention will be explained in detail, referring to examples.

Example 1.

52.6 g of sodium chloride, 3.7 g of potassiam chloride, 1.4 g of magnesium chloride, 22.2 g of sodium acetate and 50.2g of sodium gluconate were dissolved in 1,000 ml of distilled water for injection thereby to prepare an aqueous electrolyte solution, and the resulting solution was diluted to 8 l. 500 g of polyoxyethylene-polyoxypropylene copolymer having a molecular weight of 8,200 was further dissolved therein. The resulting solution was filtered, and 1.5 kg of perfluorotributylamine was added to the filtrate. The mixture was vigorously stirred with a propeller stirrer for about 30 minutes thereby to obtain a crude emulsion. Distilled water was added to the crude emulsion to make total volume 10 l. The thus obtained crude emulsion was placed in a liquid tank of Manton-Gaulin injection-type emulsifier and emulsified by injecting the emulsion under a pressure of 140 kg/cm² at the first stage, under a pressure of 500 kg/cm² at the second and third stages, under a pressure of 560 kg/cm² at the fourth stage and under a pressure of 140 kg/cm² at the fifth stage, while keeping the temperature at 40° to 50°C.

The total amount of the thus obtained emulsion was passed through De Laval type centrifuge, type BK15K (motor: 1,500 rpm; rotor: 9,000 rpm) at a flow rate of 30 l/hr for about 20 minutes, and the supernatant emulsion was collected. However, 500 ml of the initially passed emulsion was returned to the centrifuge because of poor centrifuging effect, and recentrifuged. By the centrifugal operation, about 8 l of the emulsion containing 15 W/V % of fluorocarbon was obtained. The thus obtained emulsion was fractioned in injection vials, and the vials were plugged and placed in a rotary sterlizer. The emulsion was sterilized at 115°C under rotation at 12 rpm for 15 minutes. The emulsion after the rotary sterilization contained about 90% of fluorocarbon having particle sizes of 0.05μ to 0.25μ and about 10% of that having particle

sizes of 0.25μ to 0.375μ, but contained no fluorocarbon having larger particle sizes. The oxygen content of the thus obtained emulsion was 9.3 ml/l.

Example 2.

1.5 g of perfluorotributylamine was heated and boiled at 100°C to drive the dissolved gas out, and cooled to room temperature by passing oxygen therethrough. Further, perfluorotributylamine was subjected to pressure reduction to 40 mm Hg at 5°C to drive the dissolved gas out, and the dissolved gas was replaced with oxygen gas. Emulsification was carried out in the same manner as in Example 1, except the thus obtained perfluorotributylamine was used and all the operations were conducted under the oxygen atmosphere, whereby an emulsion having the same particle size distribution as in Example 1 and an oxygen content of 52.8 ml/l was obtained.

Example 3.

40 g of polyoxyethylene-polyoxypropylene copolymer having a molecular weight of 10,500 was dissolved in 800 ml of Ringer's solution containing sodium lactate. The thus obtained solution was filtered, and 100 g of perfluorobutyltetrahydrofuran was added to the filtrate. The resulting mixture was stirred at room temperature in a homomixer for 15 minutes, whereby a crude emulsion was obtained. The Ringer's solution containing sodium lactate was further added to the crude emulsion to make the total volume 1l. 100 ml each of the crude emulsion was emulsified in a magnetostrictive ultrasonic generator at 19 KC for 15 minutes, while keeping the temperature at 40°C or less. The thus obtained emulsions were collected and placed in a Saval type continuous centrifuge (the word SAVAL is a Trade Mark). The emulsion was centrifuged at 1000 x g by passing all the amount of the emulsion therethrough for 10 minutes, whereby about 1 l of emulsion containing about 8 W/V % of fluorocarbon was obtained. The thus obtained emulsion was sterlized under rotation under the same conditions as in Example 1, whereby an emulsion having almost same particle size distribution as in Example 1 and an oxygen content of 8.7 ml/l was obtained.

Example 4.

Emulsification was carried out in the same manner as in Example 3, except that the perfluorobutyltetrahydrofuran deaerated under a reduced pressure whose dissolved gas was replaced with oxygen in the same manner as in Example 2 was used and all the operations were conducted under the oxygen atmosphere, whereby an emulsion having almost the same particle size distribution as in Example 1 and an oxygen content of 47.8 ml/l was obtained.

Example 5.

One kg of purified yolk lecithin was emulsified in 40 l of an aqeuous electroylte solution containing 0.9 g of sodium chloride, 1.94 g of potassium chloride, 2.24 g of sodium lactate, 0.142 g of magnesium chloride and 10 g of sorbitol in 1 l of distilled water and the resulting emulsion was filtered. Then, 5 kg of perfluoro-octane was added to the filtrate, and the mixture was vigorously stirred by a propeller stirrer, thereby to prepare a crude emulsion. The aqueous electrolyte solution having said composition was further added to the crude emulsion to make the total volume 50 l, and the emulsification, centrifuging and rotary sterilization of the crude emulsion were carried out in the same manner as in Example 1, whereby about 40 l of an emulsion containing 10% of fluorocarbon having particle sizes of 0.05μ and 0.375μ and an oxygen content of 7.3 ml/l was obtained.

Example 6.

40 kg of perfluorotributylamine was placed in a vessel capable of withstanding a pressure reduction and subjected to pressure reduction to 10 mm Hg while cooling it to 0°C. The vessel was vibrated occasionally and kept to the pressure reduction for at least 20 minutes to drive the dissolved gas out of the perfluorotributylamine completely. All the following operations were carried out carefully in the oxygen atmosphere. Then, oxygen gas was introduced into the perfluorotributylamine liquid to return the pressure reduction to the atmospheric pressure and dissolve the oxygen in the liquid. The thus obtained liquid was added to 195 l of physiologically saline water containing 4 W/V % of polyoxyethylene-polyoxypropylene copolymer having a molecular weight of 10,500, which were heated to 100°C in advance to drive the dissolved gas therefrom and then saturated with oxygen gas, and the resulting mixture was vigorously stirred for about 30 minutes by a propeller stirrer thereby to obtain a crude emulsion. The thus obtained crude emulsion was placed in a liquid tank of a Manton-Gaulin injection type emulsifier and emulsified by injecting the emulsion under a pressure of 140 kg/cm^2 at the first stage, under a pressure of 500 kg/cm^2 at the second and third stages, under a pressure of 560 kg/cm^2 at the fourth stage and under a pressure of 140 kg/cm^2 at the fifth stage, while keeping the temperature at 40° to 50°C.

The total amount of the resulting emulsion was passed through a De Laval type centrifuge, type BP15K (motor: 1,500 rpm; rotor: 9,00 rpm) at a flow rate of 30 l/hr for about 20 minutes, and the supernatant emulsion was collected. However, 500 ml of the initially passed emulsion is returned to the centrifuge because of the poor centrifuging effect and recentrifuged. By the centrifuging operation,

about 200 l of an emulsion containing 20 W/V % of fluorocrabon was obtained. The resulting emulsion was fractioned into injection vials in the oxygen atmosphere, and the vials were plugged and placed in a rotary sterilizer, where sterilization was carried out at 121°C for 15 minutes under rotation at 18 rpm. The resulting emulsion after the rotary sterilization had about 90 W/V % of fluorocarbon having particle sizes of 0.05μ to 0.25μ and about 10 W/V % of that having particle sizes of 0.25μ to 0.375μ, but contained no fluorocarbon having larger particle sizes. The oxygen content of the emulsion was 69.0 ml/l.

The present inventors exchanged blood of dogs and monkeys with the emulsions prepared in the foregoing examples until the hematocrit value reached 3%, and 18 dogs and 7 monkeys, whose almost entire blood was successfully exchanged with the emulsions by operational procedure based on transfusion, could survived normally for three months, and after three months, they were sacrificed and dissected, but no abnormal state was observed throughout all the tissues. It was recognized from that result that the fluorocarbon emulsion whose particle sizes were adjusted to 0.05μ to 0.375μ had an ability to carry oxygen and carbon dioxide through the living body as a substitute for red blood-cells.

WHAT WE CLAIM IS:—

1. A process for preparing an injectable fluorocarbon emulsion capable of carrying oxygen, which comprise emulsifying in an aqueous salt solution with surfactant a fluorocarbon selected from perfluorobutyltetrahydrofuran, perfluorotributylamine, perfluorooctane, perfluorodecalin, perfluoromethyldecalin and 2 - monohydroxy - nonacosafluoro - 3,6,9,12-tetraoxa - 5,8,11 - trimethylpentadecane, the fluorocarbon having an ability to dissolve at least 30 V/V % oxygen under a 100% oxygen atmosphere at an atmospheric pressure, centrifuging the resulting aqueous emulsion to adjust the particle size of fluorocarbon particles in the emulsion to within the range of 0.05μ to 0.25μ, and sterilizing the resulting emulsion under rotation.

2. A process according to Claim 1, wherein the surfactant is a polyoxyethylene-polyoxypropylene copolymer having a molecular weight of 8,200 to 10,500 or a yolk lecithin or a soybean lecithin.

3. A process according to Claim 1 or 2, wherein particles of the emulsion after the rotary sterilization have particles sizes of 0.05μ to 0.375μ.

4. A process according to Claim 1, 2 or 3 wherein the rotary sterilization is carried out at 115°C at 12 rpm for 15 minutes or at 121°C at 18 rpm for 15 minutes.

5. A process according to any of Claims 1 to 4, wherein the aqueous emulsion is deaerated under a reduced pressure and blown with pure oxygen gas before the centrifuging and the successive operations are all carried out in an oxygen atmosphere thereby to obtain an emulsion having a higher oxygen content.

6. A process according to any of Claims 1 to 4, wherein the fluorocarbon and the aqueous salt solution are deaerated under a reduced pressure and oxygen is blown thereinto dissolve oxygen therein in advance, and all the operations are carried out in an oxygen atmosphere.

7. A process for preparing an injectable fluorocarbon emulsion substantially as described in any one of the Examples herein.

8. An injectable fluorocarbon emulsion prepared by a process according to any preceding claim.

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